Expression of Neprilysin in Skeletal Muscle Reduces Amyloid Burden in a Transgenic Mouse Model of Alzheimer Disease

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Neprilysin (NEP) is a zinc metallopeptidase that efficiently degrades the amyloid β (Aβ) peptides believed to be involved in the etiology of Alzheimer disease (AD). The focus of this study was to develop a new and tractable therapeutic approach for treating AD using NEP gene therapy. We have introduced adeno-associated virus (AAV) expressing the mouse NEP gene into the hindlimb muscle of 6-month-old human amyloid precursor protein (hAPP) (3X-Tg-AD) mice, an age which correlates with early stage AD. Overexpression of NEP in muscle decreased brain soluble Aβ peptide levels by ~60% and decreased amyloid deposits by ~50%, with no apparent adverse effects. Expression of NEP on muscle did not affect the levels of a number of other physiological peptides known to be *in vitro* substrates. These findings demonstrate that peripheral expression of NEP and likely other peptidases represents an alternative to direct administration into brain and illustrates the potential for using NEP expression in muscle for the prevention and treatment of AD.

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INTRODUCTION

Alzheimer disease (AD), the major form of dementia in the elderly, afflicts more than 26 million people worldwide. The cause of AD, although still not fully understood, is believed to involve an accumulation of amyloid β (Aβ) peptides and the subsequent destruction of neurons by Aβ aggregates.[1]

Neprilysin (NEP) is a metalloendopeptidase that functions to degrade peptides in brain and peripheral tissues. NEP is one of the major Aβ-degrading enzymes, and its overexpression in brain can both prevent and clear Aβ deposits in mouse models of AD.[2,3] Therefore, efforts to use NEP therapeutically for treating AD have recently received attention but have been limited to viral-mediated NEP gene expression in brain or the implantation of cells expressing the NEP gene in the brain.[3,4]

It has been established that there is a dynamic equilibrium involving receptor-mediated transport of Aβ into and out of the brain.[5,6] Altering this equilibrium leads to Aβ redistribution.[7–9] For example, passive immunization with Aβ antibodies caused a lowering of brain Aβ presumed to be due to the binding of plasma Aβ in immune complexes resulting in a net efflux of Aβ from the brain and preventing Aβ influx from plasma into brain.[8,10] The finding that peripherally administered antibodies can enter the brain suggests that a part of the effect of passive immunization could be due to a central nervous system effect.[11] Other Aβ-binding compounds, gelsolin and GM1, also influence the shift of Aβ between central nervous system and plasma.[12] Similarly, peripheral administration of the Nogo-66 receptor for myelin inhibitory proteins increased serum Aβ and improved cognitive function in hAPP transgenic mice.[13] Sagare *et al.*[14] showed that a soluble form of the low-density-lipoprotein-receptor-related protein when injected peripherally into hAPP mice produced a dramatic 60–90% reduction in brain Aβ and improved learning, spatial memory, and recognition further suggesting that clearance of plasma Aβ can alter brain Aβ.

In the present study, we have focused on a gene therapy strategy for the treatment of AD involving adeno-associated virus (AAV)–mediated expression of the Aβ-degrading peptidase NEP in muscle. Because muscle is in contact with blood and because NEP is a cell surface protease, it was anticipated that muscle NEP would clear plasma Aβ and in turn lower the brain Aβ burden by altering Aβ transport dynamics. Our findings reveal that this is indeed the case, which is discussed further.

RESULTS

AAV-mediated NEP expression in skeletal muscle

We initially determined whether mouse NEP (mNEP) could be efficiently expressed in mouse skeletal muscle via AAV8 gene delivery. One month after intramuscular injection of varying amounts of NEP-AAV8, NEP expression was produced in a dose-dependent manner as measured by western blot analysis (**Figure 1**). It is worth noting that endogenous muscle NEP was detectable and was determined to be at a level of ~8 ng NEP

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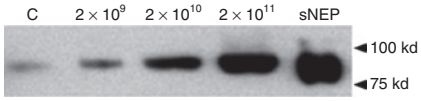

Figure 1 Expression of mouse NEP in mouse limb muscle is dependent on the dose of NEP-AAV8. Nine-month-old 3X-Tg-AD mice were injected with the indicated amount of NEP-AAV8 viral genomes (vg) into the hindlimb. Injections were 5 µl per site using six sites in the hindlimb muscle. Six weeks post injection, the mice were killed, the muscle dissected, and homogenates prepared in phosphate-buffered saline. Western blot analysis was performed using 50 µg of muscle homogenate and goat anti-mouse neprilysin. C, untreated 3X-Tg-AD mice; NEP, neprilysin; sNEP, 50 ng of purified NEP.

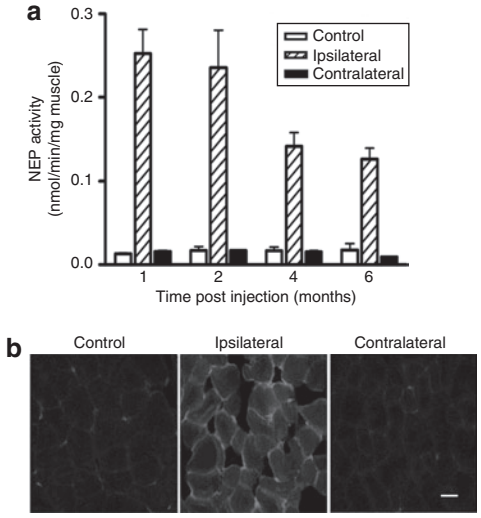

Figure 2 NEP expression in muscle mediated by AAV8 is sustained post injection. (**a**) NEP activity in muscle 4 months post injection of NEP-AAV8. Nine-month-old 3X-Tg-AD mice were injected with 2 × 10^{11} viral genomes (vg) of either mNEP-AAV8 or GFP-AAV8 into six sites within the hindlimb (5 µl per site), with the contralateral hindlimb untreated. Mice were killed at the indicated times post injection, the muscle dissected out, and used to measure NEP activity. Data are presented as mean ± SEM. (n = 3). (**b**) Immunohistochemistry of NEP in muscle. Sections of muscle from mice at 4 months post injection with 2 × 10^{11} vg NEP-AAV8 were stained with anti-mouse NEP antibody. Scale bar presents 100 µm. NEP, neprilysin.

per mg hindlimb muscle, whereas AAV-mediated NEP expression produced levels >90 ng NEP per mg muscle.

We next determined whether the expression of NEP in hindlimb muscle can be sustained. For this analysis, we injected a single dose of 2 × 10^{11} viral genomes (vg) of NEP-AAV8 into the hindlimb muscle of 3X-Tg-AD mice. At 1, 2, 4, and 6 months post injection, mice were killed and muscle homogenates prepared and analyzed for NEP activity with the synthetic substrate glutaryl-Ala-Ala-Phe-MNA. As shown in **Figure 2a**, NEP expression was sustained for 6 months, although it appeared to decrease from a rate of 0.25 nmol glutaryl-Ala-Ala-Phe-MNA hydrolyzed/minute/mg muscle to 0.13 nmol glutaryl-Ala-Ala-Phe-MNA hydrolyzed/minute/mg muscle after 6 months. Only endogenous NEP activity (0.014 nmol glutaryl-Ala-Ala-Phe-MNA hydrolyzed/minute/mg muscle) was detected in the contralateral muscle. The expressed NEP was easily detected on muscle cells by immunohistochemistry (**Figure 2b**). We next determined

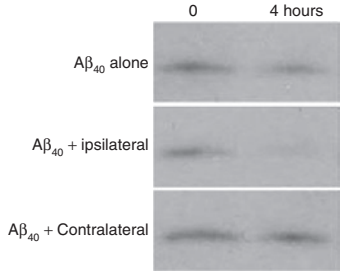

Figure 3 Neprilysin (NEP) expressed on muscle is active toward degrading Aβ. Aβ_{1-40} (2 µmol/l) was incubated with muscle homogenates (2.2–2.3 µg protein) for 4 hours in 100 µl phosphate-buffered saline buffer. Samples were then run on an SDS-Tricine gel and Aβ_{1-40} detected by western blot analysis with monoclonal antibody 6E10. Note, in the ipsilateral muscle expressing NEP, the Aβ is mostly cleaved, whereas no detectable cleavage was observed with the control (contralateral) muscle homogenates.

whether the expressed NEP was active toward Aβ. To accomplish this, we generated muscle homogenates from the ipsilateral NEP-AAV8 injected muscle and compared its ability to degrade Aβ to the contralateral uninjected hindlimb. As shown in **Figure 3**, under the conditions of this experiment, the NEP-AAV8 injected muscle homogenate hydrolyzed Aβ, whereas the control contralateral muscle homogenate did not cleave Aβ at a detectable rate. Although not shown, the same amount of purified NEP activity produced a similar extent of Aβ degradation as obtained with the ipsilateral muscle homogenate.

Gene delivery of NEP in muscle decreases brain Aβ levels

In order to determine whether peripheral expression of NEP in mouse hindlimb can lower brain Aβ levels, NEP-AAV8 or GFP-AAV8 (2 × 10^{11} vg) was injected into the hindlimb of 6-month-old 3X-Tg-AD mice (n = 7 per group). It has been reported that intracellular Aβ immunoreactivity initially appears in the cortex and CA1 field of the 6-month-old 3X-Tg-AD mouse and that extracellular Aβ deposits are evident in cortex and hippocampus by 12 months.[15] Therefore, we picked ~6-month-old 3X-Tg-AD mice for these studies, as they would best represent early stages of AD. These mice were killed 6 months after treatment when they reached an age of 12 months old, and their muscle and brains were collected for analysis. **Figure 4a** shows that only mice receiving NEP-AAV8 expressed high levels of NEP activity in their hindlimb muscle. Soluble brain Aβ in this treated group was reduced by ~60% (P = 0.0025) compared with the control group (**Figure 4b**).

We measured and quantified Aβ deposits in brain sections to further assess the effect of peripheral NEP expression on brain amyloid load. **Figure 4c** shows immunohistochemical staining for Aβ and hAPP of control untreated mice compared to AAV8-NEP–treated mice. Quantitation of the data in **Figure 4d** shows that the amyloid burden was decreased >50% in the treated mice (25,610 ± 3,834 pixels/section for NEP-expressing mice versus 53,250 ± 10,050 pixels/section for control mice; P = 0.02). In contrast to the change in amyloid burden, we found that hAPP immunoreactivity levels in the hippocampus did not change with peripheral NEP expression (5,377 ± 1,142 pixels/section for NEP-expressing mice versus 6,009 ± 1,370 pixels/section for control

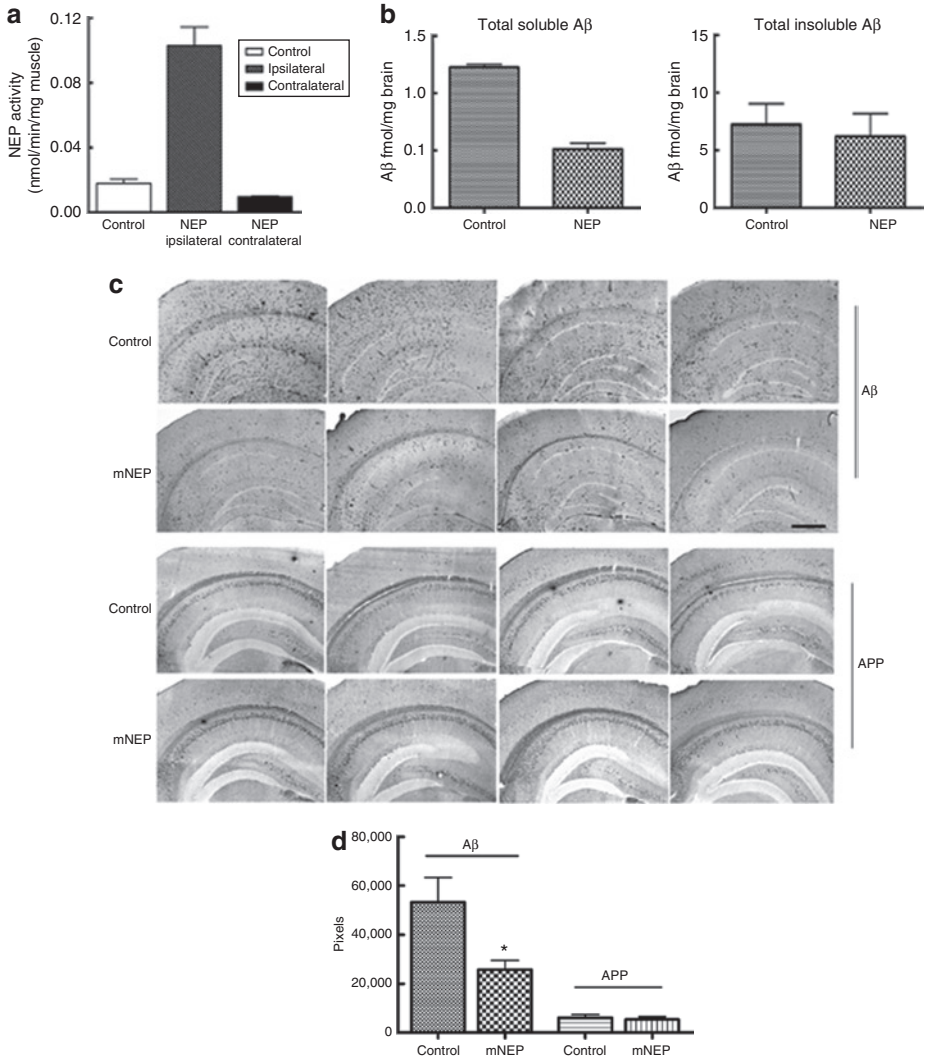

Figure 4 Effect of hindlimb muscle NEP expression on brain Aβ. At ~6 months of age, 3X-Tg-AD mice ($n = 7$) were injected in one hindlimb muscle with 2×10^{11} viral genomes (vg) of NEP-AAV8. When the mice reached 12 months of age, they were killed and analyzed for NEP expression and Aβ levels. (**a**) NEP expression in mouse hindlimb 6 months post injection. Hindlimb muscle was homogenized in phosphate-buffered saline and used to measure NEP activity as in **Figure 1**. Data are presented as mean ± SEM. ($n = 7$). (**b**) Aβ in mouse brain 6 months post intramuscular injection of NEP-AAV8. Aβ levels in 3X-Tg-AD mouse brain 6 months following intramuscular injection of NEP-AAV8 (2×10^{11} vg) decreased as determined by sandwich enzyme-linked immunosorbent assay. Data are presented as mean ± SEM. ($n = 7$). Comparing the means of NEP-treated versus control mice for Aβ, the P value is 0.0025. Due to multiple testing, a Bonferroni correction was applied and a significance level of 0.025 was used. By this criteria, the lower brain Aβ retained its statistical significance in the NEP-expressing mice. (**c**) Aβ and hAPP immunohistochemistry in the brain of mice expressing hindlimb NEP. Brain sections were stained with anti-Aβ42 and anti-APP–specific antibodies and developed using DAB. Aβ42 and APP were measured in adjacent sections of cortex and hippocampus, $n = 7$. Scale bar is 500 µm. (**d**) Quantitative evaluation of Aβ deposits in hippocampal and cortical regions. The data from **c** was quantified as described in Materials and Methods. NEP-expressing mice show reduced Aβ deposits versus control green fluorescent protein–expressing mice, $^*P < 0.05$, $n = 7$. APP, amyloid precursor protein; mNEP, mouse neprilysin.

mice, $P = 0.73$). This rules out the likelihood that the observed reduction in brain Aβ is caused by an effect on APP. Brain sections were also stained with thioflavine S to further analyze the formation of Aβ plaques (**Figure 5**). It was observed that few mature plaques appeared in the 3X-Tg-AD mice at the age of 12 months, whereas mature plaques were more abundant in the brain of a 17-month-old 3X-Tg-AD mouse. It appeared there was little, if any, change in the thioflavine S staining of the NEP-treated mice versus untreated mice. This is consistent with the lack of a significant change in total insoluble brain Aβ levels as shown in **Figure 4b**.

Gene delivery of NEP in muscle does not affect other peptides

NEP is known to hydrolyze peptides other than Aβ, which include peptides known to regulate blood pressure such as bradykinin, endothelin, and angiotensin. We thus tested the effect of NEP hindlimb expression on systolic blood pressure at 1, 2, and 4 months post injection of 2×10^{10} vg and 2×10^{11} vg NEP-AAV8. No effect was observed (**Figure 6**). We also directly measured the effect of peripheral NEP overexpression on three NEP peptide substrates, leucine-enkephalin, bradykinin, and substance P in both brain and plasma. As shown in **Figure 7**, there was no

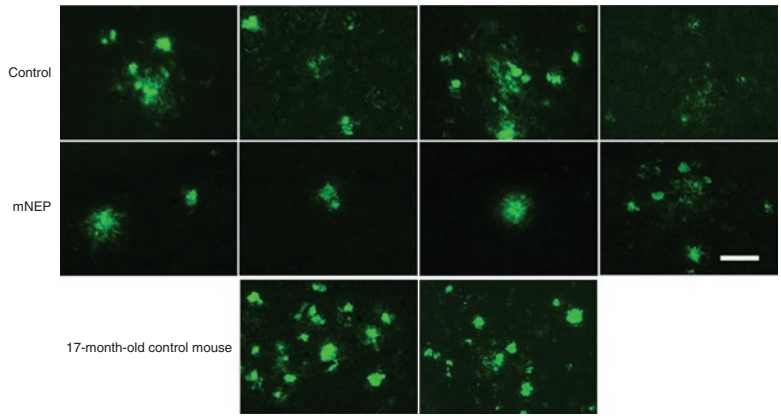

Figure 5 Thioflavine S staining of plaques in brain hippocampus. Mouse brains were stained with thioflavine S as described in Materials and Methods. Few mature plaques are seen in the 12-month-old 3XTg-AD mouse brain compared to a 17-month-old 3XTg-AD mouse brain, and little difference between NEP-treated and -untreated mice was observed. mNEP, mouse neprilysin. Bar = 50 µm.

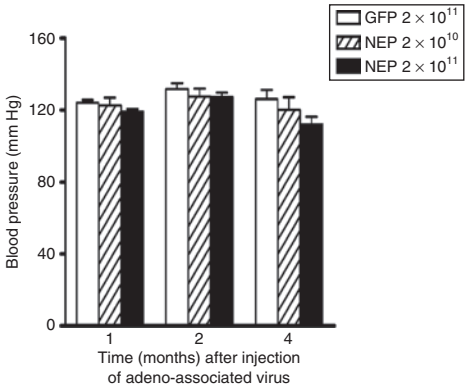

Figure 6 NEP expression in the mouse hindlimb does not affect blood pressure. Nine-month-old 3X-Tg-AD mice were injected with the indicated number of viral genomes of either mNEP-AAV8 or GFP-AAV8 into hindlimb as described in **Figure 3**. Systolic blood pressure was measured as described in Materials and Methods at 1, 2, and 4 months post injection. Data are presented as mean ± SEM (n = 9 for 2 × 10^{11} viral genomes of AAV-GFP and NEP, n = 5 for 2 × 10^{10} vg of AAV-NEP). No statistically significant difference between any groups was noted. GFP, green fluorescent protein; NEP, neprilysin.

significant change in the level of these peptides in either plasma or brain in the NEP-treated mice. We also measured plasma Aβ levels (**Figure 7**) and found it to be reduced by ~30%, but due to the low level of plasma Aβ, this difference did not reach statistical significance (P = 0.09).

DISCUSSION

The ability of NEP to degrade Aβ in brain has received considerable attention as a target for treating AD. To date, efforts to use NEP for treating AD have been limited to brain NEP gene expression mediated by virus or cells introduced into the brain.[3,4] We previously showed a reduction of Aβ burden in brain by expression of NEP on the surface of leukocytes.[16] NEP expression on leukocytes was achieved by transduction of bone marrow cells with NEP-lentivirus, followed by bone marrow transplantation. Here we show that a noninvasive, more tractable approach of expressing NEP in muscle can also lower brain Aβ and prevent Aβ deposition. In this study, we tested 6-month-old 3X-Tg-AD mice as to the extent that these mice reflect the accumulation of Aβ that occurs in AD, this age represents the equivalent of detectable early stage AD, a time when treatment would likely be initiated. The decline in brain Aβ observed in this study should translate to a decline in Aβ accumulation in the AD patient brain, and a delay or amelioration of the disease.

The mechanism whereby peripheral NEP reduces brain Aβ burden is likely to involve degradation of blood-borne Aβ by NEP on the surface of muscle cells as blood comes in contact with the muscle. Although a statistically significant decrease in plasma Aβ was not observed, a trend toward lower Aβ levels was seen. The rather low level of plasma Aβ in these mice makes these measurements less precise and contributes to scatter in the data. As plasma Aβ is hydrolyzed by NEP, it is replenished by peripherally derived Aβ as well as by the efflux of brain Aβ, primarily via the low-density-lipoprotein-receptor-related protein 1. Hydrolysis of plasma Aβ by peripheral NEP would also lower brain Aβ by preventing reuptake via the receptor for advanced glycation end products. This would change blood-brain transport dynamics from a bidirectional process to a unidirectional process with the net effect of decreased brain Aβ as observed in this study. In unpublished studies (Y. Liu, C. Studzinski, T. Beckett, M.A. Hersh, M.P. Murphy, R. Klein *et al.*, unpublished results), we demonstrated that AAV virus injected into muscle does not express NEP mRNA in the brain, thus showing that it is indeed peripheral NEP that lowers brain Aβ.

A similar phenomenon is proposed to occur when using reagents that bind Aβ such as anti-Aβ antibodies, Aβ-binding proteins such as soluble receptor for advanced glycation end products and soluble low-density-lipoprotein-receptor-related protein 1, or other reagents that bind Aβ such as gelsolin or ganglioside GM1.[8,10,12,14] The effect produced by reagents that bind Aβ has been termed a "sink effect." We suggest that peripheral hydrolysis by NEP and other peptidases be termed the "drain effect," to note that the peripheral Aβ is not accumulating as in a sink, but rather is being destroyed as if going down a drain.

The observation that, of the peptides tested, only brain Aβ levels were affected by peripherally expressed NEP is likely related to the fact that blood-derived Aβ arises via an active transport system, whereas other blood-borne peptides are not actively

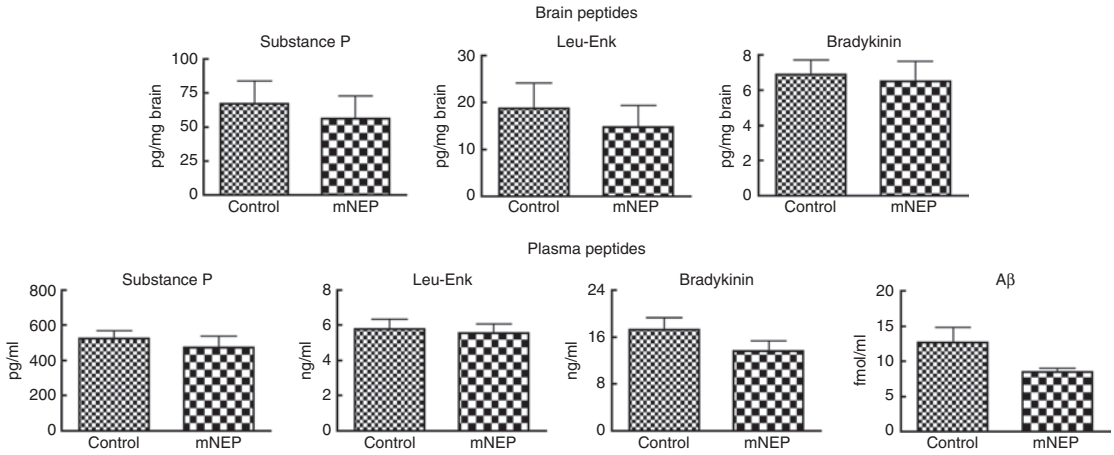

Figure 7 NEP expression in the mouse hindlimb does not affect the level of substance P, leucine-enkephalin, and bradykinin in brain or blood. Commercial ELISA kits were used to measure substance P, bradykinin, and leucine-enkephalin in brain or plasma samples prepared as described in Materials and Methods. Plasma Aβ levels were measured as described in Methods. No statistically significant difference between any groups was noted. Leu-Enk, leucine-enkephalin; mNEP, mouse neprilysin.

transported from the brain. Additionally, there are peptidases in blood that degrade these peptides, but do not degrade Aβ, at a significant rate. These include carboxypeptidases, angiotensin-converting enzyme, and aminopeptidases.[17]

In summary, these studies suggest that the use of muscle expressed NEP via AAV gene transfer may be an effective way to lower brain Aβ levels in AD patients. AAV is attractive for gene delivery because this virus can produce long-term expression without pathogenicity[18] and is currently being tested as a delivery vehicle in human gene therapy. NEP is an attractive enzyme to use because it efficiently lowers brain Aβ, yet did not affect the levels of other peptides.

MATERIALS AND METHODS

Generation of AAV vectors. The expression cassette incorporated the cytomegalovirus/chicken β-actin hybrid promoter to drive transgene expression. The cassette was flanked by AAV2 terminal repeats. The constructs, either for mouse NEP or green fluorescent protein (GFP), were cross-packaged into AAV8 vectors as described.[19] Three days after transfecting human embryonic kidney 293-T cells with transgene plasmid along with helper and packaging plasmids for AAV8, cells were harvested, lysed, and applied to a discontinuous gradient of iodixanol.[19] The AAV fraction was washed and concentrated on Millipore Biomax 100 Ultrafree-15 units (Millipore, Billerica, MA) and then sterilized with Millipore Millex-GV syringe filters. Virus preparations were titered for encapsulated vg) by dot-blot.[19]

Treatment of mice. The 3X-Tg-AD mouse model of AD[7] was used in this study. Mice received injections of 5 µl per site of NEP-AAV8 or GFP-AAV8 into six sites per hindlimb muscle. At 6 months post injection, mice were killed, the muscle dissected, and homogenates prepared in phosphate-buffered saline (PBS) for NEP assay. At the time of killing, brains were removed and placed on ice. One half-brain was used for peptide measurements and the other half-brain was frozen for sectioning and immunohistochemistry. All procedures were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the University of Kentucky Institutional Animal Care and Use Committee.

NEP activity measurements. Mouse muscle was homogenized at 1 mg/ml in ice-cold PBS using an Ultra-Turrax T-25 basic homogenizer (IKA, Wilmington, NC). The homogenate was centrifuged at 1,000 *g* for 20 minutes and the supernatant was then centrifuged at 100,000 *g* for 1 hour. The pellet containing NEP was resuspended in PBS and used for the determination of NEP activity. Glutaryl-Ala-Ala-Phe-4-methoxy-2-naphthylamide (Glutaryl-Ala-Ala-Phe-MNA; Sigma, St Louis, MO), which is cleaved by NEP to produce Phe-4-methoxy-2-naphthylamide, was routinely used as the substrate. The latter was then cleaved by an aminopeptidase to produce the fluorescent 4-methoxy-2-naphthylamine. Reaction mixtures (200 µl) contained 100 µmol/l glutaryl-Ala-Ala-Phe-4-methoxy-2-naphthylamide, 5 µl of muscle membrane fraction, 1 µg of purified recombinant human puromycin-sensitive aminopeptidase[20] and 20 mmol/l MES buffer, pH 6.5. Reactions were initiated by the addition of the NEP containing membrane fraction and monitored continuously at 37 °C at an excitation wavelength of 340 nm and an emission wavelength of 425 nm. Phosphoramidon (50 µmol/l), an inhibitor of NEP, reduced the observed activity by >90%. A standard curve relating fluorescence change to the amount of 4-methoxy-2-naphthylamine was used to calculate rates. The specific activity of NEP is expressed as nmol product formed/minute/mg protein and represents an average of at least two separate measurements performed in duplicate.

For measurements of Aβ cleavage, $A\beta_{1-40}$ (2 µmol/l) was incubated with muscle homogenates (2.2–2.3 µg protein) for 4 hours in 100 µl PBS buffer. Samples were then run on an SDS-Tricine gel and $A\beta_{1-40}$ was detected with monoclonal antibody 6E10.

Protein concentration was measured using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL).

Western blot analysis. Muscle homogenates (50 µg) were subjected to SDS-PAGE and western blot analysis using a goat anti-mouse NEP antibody (R&D Systems, Minneapolis, MN) and horseradish peroxidase–conjugated anti-goat IgG. A secreted form of NEP was purified from human embryonic kidney 293 cells[21] and used as a control in western blot analysis.

Blood pressure measurements. Systolic blood pressure was measured by the tail-cuff method (BP-2000; Visitech Systems, Apex, NC).[22] Mice were conscious, but restrained on a 37 °C heated stage. Measurements were taken on five consecutive days at the same time of day each time. Mice were subjected to 10 preliminary and 10 recorded measurements.

Brain Aβ measurements. Each half-brain was homogenized in radioimmunoprecipitation assay buffer (50 mmol/l Tris-HCl, 150 mmol/l NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, and a protein inhibitor cocktail (Roche Diagnostics, Indianapolis, IN), pH = 8.0. The homogenate

was centrifuged at 20,000 *g* for 30 minutes and the supernatant taken for Aβ measurements. Ab9 (anti-$A\beta_{1-16}$)[23] was used as the capture antibody and 4G8 (anti-$A\beta_{17-24}$; Signet Laboratories, Dedham, MA) as the detection antibody for total Aβ analysis.

Peptide measurements. For measuring substance P, bradykinin, and leucine-enkephalin levels in the brain, one-fourth of each brain was homogenized in 5 mol/l guanidine-HCl in PBS at pH 8.0 containing a protease inhibitor cocktail. After stirring overnight at room temperature, the samples were diluted 10 times into pH 7.4 Dulbecco's PBS, containing 5% bovine serum albumin and 0.03% Tween-20, and centrifuged at 16,000 *g* for 20 minutes at 4 °C. The supernatants were analyzed with commercial enzyme-linked immunosorbent assay kits for substance P (Assay Designs, Ann Arbor, MI), bradykinin, and leucine-enkephalin (Phoenix Pharmaceuticals, Burlingame, CA). For measuring plasma peptides, 1 ml of blood was collected in 30 µl of PBS containing 1,000 U/ml of heparin. The plasma was collected by centrifugation at 1,000 r.p.m. in an Eppendorf centrifuge for 20 minutes and used for enzyme-linked immunosorbent assay analysis.

Immunohistochemistry and Aβ quantitation. Cryostat coronal sections (16 µm) from frozen brains were fixed in 3% paraformaldehyde in PBS. Following incubation with 3% H_2O_2 to quench endogenous peroxidase activity, the sections were blocked with PBS containing 0.1% Triton X-100, 0.1% bovine serum albumin, and 2% normal horse serum for 1 hour at room temperature and then incubated overnight at 4 °C with an mAb against the N-terminus of Aβ42 (Ab26-2.13)[24] or goat anti-APP R8666 antibody, obtained from Maria Kounnas (Torrey Pines Pharmaceutical, La Jolla, CA). Sections were washed in PBS three times and incubated with the appropriate secondary antibody, horse anti-mouse conjugated to peroxidase (1:2500; Vector Laboratories, Burlingame, CA) or horse anti-rabbit conjugated to peroxidase (1:2500; Vector Laboratories), for 1 hour at room temperature. Sections were developed with DAB (Vector Laboratories).

Thioflavine S staining was performed according to Oddo *et al.*[25] Briefly, brain sections were incubated in a 0.5% solution of thioflavine S (Sigma-Aldrich, St Louis, MO) in 50% ethanol for 10 minutes. The sections were then washed twice in 50% ethanol and water, respectively.

Sections of muscle (30 µm) were fixed in 3% paraformaldehyde in PBS, and blocked with PBS containing 0.1% Triton X-100, 0.1% bovine serum albumin, and 2% normal horse serum for 1 hour at room temperature after which they were washed in PBS. The muscle sections were then incubated with a primary antibody goat anti-mouse NEP antibody (R&D Systems), followed by the secondary antibody, anti-goat conjugated with Red Texas (Vector Laboratories).

Quantitative analysis of Aβ deposit load was performed as described by Oddo *et al.*[7] Briefly, serial sections of each brain (six sections per animal, seven animals per group) were stained with appropriate antibodies. Images of stained sections were captured using an Olympus Provis AX80 microscope (B&B Microscopes, Philadelphia, PA) and imported into Image-Pro Plus 6.2 software (Media Cybernetics, Bethesda, MD). The area covered with Aβ deposits or hAPP in the hippocampus was measured and the pixel density of Aβ- or APP-immunopositive deposits compared.

Statistical analysis. Data was analyzed by the Student's *t*-test using GraphPad Prism 4 software (GraphPad Software, La Jolla, CA). Values were considered to be statistically significant with a P value <0.05. Data are presented as mean ± SEM.

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